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Specificity, Catalysis, and Regulation: Effects of Metal Ions on Polyammonium Macrocycle Catalyzed Dephosphorylation of ATP

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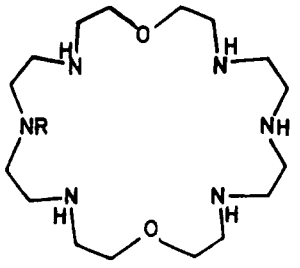
Received August 4, 1986

The catalytic influence of the combined effects of metal ions and a hexaazadioxa macrocycle, [24]-N_6O_2, on the dephosphorylation of adenosine triphosphate was examined for cadmium(II), zinc(II), magnesium(II), calcium(II), and lanthanum(III). The rates of dephosphorylation increased in that order at pH 7.6, with magnesium ion in conjunction with the macrocycle having essentially the same rate as the macrocycle alone. In the presence of magnesium, calcium, and lanthanum ions, the percentage of an intermediate phosphoramidate species was found to increase. Pyrophosphate was formed in the presence of these three metal ions after a period of 24 h at pH 4, which indicates that the metal ions can provide a regulatory effect on the catalytic reaction, by altering the reaction pathway. Metal ions had less of an influence on adenosine diphosphate. Rate studies were made by using ^{31}P NMR, and metal ion, macrocycle, and ATP interactions were assessed by using ^{31}P and ^{13}C NMR techniques. Rate retardations for zinc(II) and cadmium(II) were attributed to competition between these metal ions and the nucleotide for the macrocycle. The rate enhancements observed for calcium(II) and lanthanum(III) were linked to decreased tendencies of these metal ions to interact with the macrocycle and to increased charge for the latter ion.

Introduction

The universal requirement of metal ions in enzymatically assisted dephosphorylation of adenosine 5′-triphosphate and related nucleotides has resulted in scientific focus on the mechanistic implications of metal ions and their complexes.[1] While polyamines also possess potential as catalytic agents, in view of the propensity of polyammonium species for complexing phosphates and nucleotides,[2,3] this area has been relatively unexplored with the exception of certain linear[4,5] and more recently macrocyclic polyamines.[6-8] Although the linear polyamines show little aptitude for activating the catalytic process,[4,5] large-ring macrocyclic analogues exhibit a range of catalytic ability even in the absence of metal ions, traceable to distinct structural and electronic aspects of the macrocycle,[6,7] especially ion-pairing and hydrogen-bonding effects. Furthermore, there is evidence that the hydrolytic pathway in these macrocyclic systems involves phosphorylation of the macrocycle, yielding an intermediate phosphoramidate species that undergoes subsequent hydrolysis.[6-8]

The 24-membered macrocycle 1,13-dioxa-4,7,10,16,19,23-hexaazacyclotetracosane (**1**) has been discovered to be an exceptional mimic for enzymes that hydrolyze adenosine triphosphate (ATP), i.e. the ATPases, and similar enzymes involved in phosphoryl transfer.[6,7] Three features prominent in enzymatic catalysis



1, R = H
2, R = PO_3^{2-}

are observed: (1) *specificity*, in the high association constant for the formation of the initial protonated macrocycle ATP complex; (2) *electrostatic catalysis*, in the pH–rate profile of the hydrolysis; (3) *covalent catalysis*, in the formation of a phosphorylated macrocyclic intermediate. The latter feature, a phosphorylated enzyme intermediate, while not exhibited by all of the ATPases, does occur in several, e.g. ATP-dependent ion transport ATPase.[9] Thus, the design of models for enzymatic reactions commonly addresses specificity and catalysis, as noted for the polyammonium macrocycle cited above.[6,7]

Another feature of enzymatic reactions, *control* or *regulation*, plays a crucial role in the operation of these complex catalysts, and the potential of macrocyclic ligands in this area has been recognized since the late 1970s.[10] An excellent example of regulation in model systems is, for example, the pH regulation of divalent/monovalent metal cation transport using a specially designed crown ether, reported by Hriciga and Lehn.[11] Allosteric control is another such regulatory event utilized in biological transformations. A simple model of allosteric control is the action of a third effector molecule, or "messenger", in the activation or deactivation of an enzyme or in the modification of the chemistry of an enzyme–substrate complex to produce an alternate reaction pathway.[12]

The enzymatic pathways utilizing the ATP phosphotransferases, hydrolases, and synthetases do require the presence of metal ions, notably magnesium(II), for a variety of dephosphorylation processes in addition to the enzymatic protein framework. Thus, in an attempt to model more exactly these biological counterparts, the cumulative influence of a macrocyclic receptor in conjunction with added metal ions was examined in this laboratory.[8] The result

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Table I. First-Order Rate Constants (k_{obsd}) for the Dephosphorylation of Polyphosphate Species (0.010 M) in the Presence of [24]-N_6O_2 and/or Metal Ions at pH 7.6 and 70 °C

1	substrate	metal ion	ratio substrate:1:metal	10^3k_{obsd}, min^{-1}
–	ATP		1:0:0	0.11
+	ATP		1:1.5:0	25
+	ADP		1:1.5:0	6.8
–	ATP	Ca(II)	1:0:1.5	4.1
+	ATP	Ca(II)	1:1.5:1.5	44
+	ADP	Ca(II)	1:1.5:1.5	7.3
+	PPP	Ca(II)	1:1.5:1.5	8.7
+	PP	Ca(II)	1:1.5:1.5	1.3
–	ATP	Mg(II)	1:0:1.5	0.45
+	ATP	Mg(II)	1:1.5:1.5	23
+	ADP	Mg(II)	1:1.5:1.5	2.2
–	ATP	Zn(II)	1:0:2	8.2
+	ATP	Zn(II)	1:1.5:1.5	3.2
–	ATP	Cd(II)	1:0:2	10.0
+	ATP	Cd(II)	1:1.5:1.5	2.2
–	ATP	La(III)	1:0:0.5	2.7
+	ATP	La(III)	1:1.5:0.5	93
+	ADP	La(III)	1:1.5:0.5	9.2

was that the fourth level of catalytic mimicry, regulation, was observed. This "control" was obtained by the addition of a third species, calcium ion, incidently considered to be the "third messenger" in control of cellular metabolism and function.[12] The result was a change in the reaction pathway evident in enhanced formation of a phosphorylated macrocycle (**2**) and was coupled to the formation of a product not observed under analogous conditions in the absence of calcium, namely pyrophosphate.[8] This finding provides further evidence of the ideality of simple systems such as these for biological models.

Experimental Section

The macrocycle **1** was obtained according to previously published procedures.[7] The sodium salts of ATP and adenosine diphosphate (ADP) and the lithium potassium salt of acetyl phosphate were obtained from Aldrich Chemical Co. All other chemicals were high-purity commercial products. The ^1H NMR spectra were measured on $CDCl_3$ solutions with Me_4Si as internal standard on a Varian FT-80 or Varian XL-300 spectrometer operating at 80 or 300 MHz, respectively. The ^{13}C and ^{31}P NMR spectra were obtained on the Varian XL-300 operating at 75 and 121.4 MHz, respectively. Two-dimensional NMR studies on the Varian XL-300 with HECTOR and HOMCOR pulse sequences were used to assign ^{13}C chemical shifts for **1**. Aqueous solutions, 10% in D_2O, were used for ^{31}P spectral measurements of chemical shifts in ppm in reference to phosphoric acid (85%) as an external standard, while Me_4Si was used as an external standard for ^{13}C spectra.

Kinetic Measurements. The appropriate species, consisting of nucleotide or polyphosphate and **1** in its hexabromide form and/or the metal ion dibromides (except lanthanum, which was the trichloride) were dissolved in aqueous solutions containing 10% D_2O. The buffer EPPS (*N*-(2-hydroxyethyl)piperazine-*N'*-propanesulfonic acid) when used was 0.1 M. The pH was adjusted with NaOH (2 drops of a 1 M solution followed by dropwise addition of a 0.1 M solution). A 0.5-mL aliquot was placed in a 5-mm NMR tube and heated in the probe to 70 °C by using the variable-temperature accessory. Acquisition of proton-decoupled ^{31}P spectral data (450 scans, 6 min) was obtained at time intervals depending on the reaction rate. Dephosphorylation was monitored by the disappearance and appearance of the various phosphorus signals. The hydrolysis of the phosphorylated macrocycle was followed in a similar fashion after its formation in solution by reacting equimolar mixtures of acetyl phosphate and **1** at 50 °C.[7]

Results and Discussion

Rate Studies at pH 7.6. Titrimetric data at 25 °C and $I = 0.1$ from several sources indicate pK_{a3} for $[(\mathbf{1})H_4]^{4+}$ to range from 7.20 to 8.10 and pK_{a4} for $[(\mathbf{1})H_3]^{3+}$ to vary from 8.20 to 8.30 depending on the identity of the salt used to adjust ionic strength.[3,13,14] Thus in these studies the macrocyclic ligand is

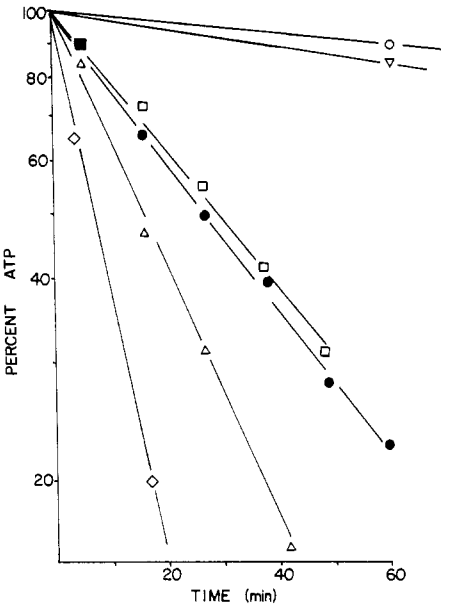

Figure 1. Semilog plot of the percentage of remaining ATP (0.010 M) vs. time (min) at 70 °C and pH 7.6 in the presence of **1** (0.015 M) alone (●) and with the following added metal ions: cadmium(II), 0.015 M (○); zinc(II), 0.015 M (▽); magnesium(II), 0.015 M (□); calcium(II), 0.015 M (△); lanthanum(III), 0.005 M (◇).

most probably a mixture of the tri- and tetraprotonated forms before complexation with either ATP or metal ions or both. The dephosphorylation of ATP at 70 °C was examined for **1** in the presence of a variety of metal ions. The rates of disappearance of ATP (Figure 1) and first order rate constants (Table I) at pH 7.6 indicate that metal ions add another dimension, that of regulation, to the catalytic reaction. Note that this catalysis is considered as dephosphorylation and not hydrolysis, due to the presence of the intermediate phosphoramidate, as discussed below. Rates varied from 0.002 min^{-1} in the presence of cadmium(II) ion to 0.093 min^{-1} for lanthanum(III). Only calcium(II) and lanthanum(III) showed distinct rate accelerations. In the presence of magnesium ion the rates compared to those for **1** alone were essentially unchanged, while cadmium and zinc slowed the reaction. Metal ions also influenced the amount of phosphoramidate intermediate, greater percentages being observed in reactions with magnesium, calcium, and lanthanum ions. The pH of the solutions varied only by 0.5 pH unit during the course of the reaction with the added metal ions in the presence of **1** and ATP (e.g., with zinc (II) and calcium(II) the pH went from 7.6 to 7.1). Buffered solutions were also run using EPPS, but the rates were diminished by a factor of 2, indicating that the buffer interfered with the catalysis. Because of the apparent interference of the buffer and the small pH shifts, rates are reported for unbuffered reactions.

The dephosphorylation of ADP to inorganic phosphate and AMP was examined in the presence of **1** and calcium(II), magnesium(II), and lanthanum(III) ions and ranged from 0.002 to 0.009 min^{-1}, for magnesium and lanthanum, respectively. The rates of dephosphorylation of tripolyphosphate and pyrophosphate were also examined for **1** plus calcium ion. Calcium was chosen because it provided acceleration of hydrolysis and is biologically relevant. While the rate of dephosphorylation for tripolyphosphate was approximately the same as that observed for ADP, the pyrophosphate reaction was considerably slower. A similar relationship of rates was previously observed for the following four species: ATP, ADP, tripolyphosphate, and pyrophosphate in the presence of **1** without added metal ion.[6,13] Thus the scope of metal ion control is greatest for ATP, the most complex of the polyphosphates studied.

A control study involving metal ion catalysis of ATP hydrolysis in the absence of macrocycle was performed under analogous

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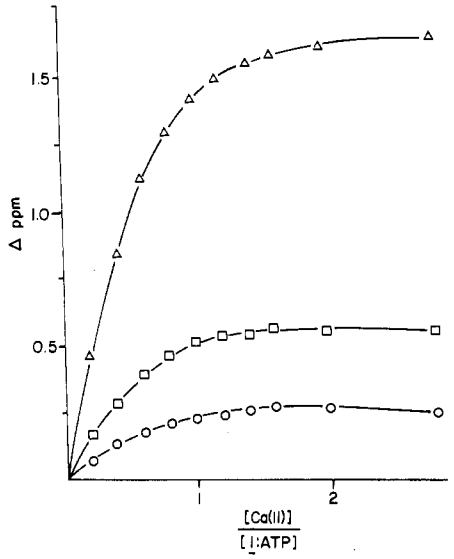

Figure 2. Titration of the binary **1**–ATP equimolar (0.010 M) mixture with calcium(II) ion at pH 7.6 using ^{31}P NMR to monitor the chemical shifts of P_α (○), P_β (△), and P_γ (□).

conditions to obtain correlatable values. The results agree with previously published data with respect to trends between the metal ions under examination[1a-f,15,18] (Table I). Interestingly, the hydrolysis rate relationships between metal ions did not necessarily correlate with the rates observed for the ternary systems. For example, while the binary zinc– and cadmium–ATP systems hydrolyzed at a faster rate than the ternary complexes (2.5- and 5-fold, respectively), the magnesium, calcium, and lanthanum binary rates were considerably retarded compared to those of the ternary systems. The pH did shift substantially during these reactions: that of zinc(II), cadmium(II), and lanthanum(II) decreased by almost 2 units; that of calcium(II) and magnesium(II) decreased by slightly over 1 unit. Again the EPPS buffer appeared to interfere with the reaction, and spectra were of very poor quality. Since the first-order rate plots were linear and these controls were only used for comparative purposes and were not the main interest of this study, no further attempts to control the pHs were made.

Binary and Ternary Complexation. The log K_s of 4.8, observed for complexation of the tetraprotonated form of **1**, $[(\mathbf{1})H_4]^{4+}$, with ATP[13] indicates high specificity between the two species. Complexation studies previously performed on the binary macrocycle–ATP system indicated the formation of a 1:1 complex.[19] Analogous techniques were used in this study to determine macrocycle:ATP:metal complexation tendencies, i.e. by monitoring ^{31}P chemical shifts during the titration of a 1:1 **1**:ATP complex with calcium ion. The results indicate that in the presence of the 1:1 complex between **1** and ATP, a 1:1:1 macrocycle:ATP:calcium(II) complex is formed (Figure 2).

Since the complicating factor in this study is the addition of metal ions to the macrocycle–ATP system, an examination of the nature of the binary interaction between ATP and the metal ions as compared to binary macrocycle:ATP and ternary macrocycle:ATP:metal relationships is necessary. A number of studies have been made to probe this aspect for binary systems of ATP and aquated metal ions.[19-22] For ATP associated with divalent calcium, magnesium, zinc, and cadmium ions, ^1H NMR has indicated that the metal ions promote stacking of the purine bases and that both zinc and cadmium exhibit association with the adenine ring.[19,22] NMR studies with lanthanum(III) and ATP indicate tris α-, β-, and γ-tridentate chelates with no evidence of binding of the metal to the adenine ring.[20,21] ^{31}P NMR studies performed in this laboratory indicate a common trend for the binary metal–ATP systems, i.e. large downfield shifts for P_β (ca. 2.5 ppm) and P_γ (between 0.75 and 1.5 ppm) and small downfield shifts for P_α (ca. 0.3 ppm). Lanthanide ion is the exception to the binary metal:ATP trends, with a relatively small downfield P_γ shift (0.38 ppm) and a small upfield shift for P_α.

The macrocycle:ATP interactions in the presence and absence of metal ion as examined by both ^{31}P NMR (Table II) and ^{13}C NMR (Table III) allow for assessment of the critical points of interaction. The macrocyclic presence results in downfield shifts

Table II. ^{31}P Chemical Shift Differences for ATP (0.01 M) in the Presence of [24]-N_6O_2 (0.015 M, **1**) and/or Metal Ions at pH 7.6 and 25 °C[a]

		Δ chem shift		
1	metal[b]	P_α[c]	P_β	P_γ
+		0.00	+0.87	+0.15
–	Ca(II)	+0.35	+2.55	+1.08
+	Ca(II)	+0.26	+2.36	+0.83
–	Mg(II)	+0.28	+2.70	+0.75
+	Mg(II)	+0.26	+2.70	+0.63
–	Zn(II)	+0.34	+2.79	+0.92
+	Zn(II)	−0.24	+0.97	−0.10
–	Cd(II)	+0.24	+2.18	+1.46
+	Cd(II)	+0.18	+0.86	+0.61
–	La(III)	−0.40	+2.54	+0.38
+	La(III)	−0.40	+2.60	−0.23

[a] Chemical shifts in ppm are given in relation to the chemical shifts for P_α (−10.60), P_β (−21.50), and P_γ (−5.98) for ATP at pH 7.6 and 25 °C. [b] La:ATP = 0.005:0.01. All other metals are 0.015:0.01 metal:ATP. [c] Chemical shifts and changes in chemical shifts are given in ppm.

Table III. ^{13}C Chemical Shift Differences for [24]-N_6O_2 (0.010 M) in the Presence and Absence of ATP (0.010 M) and/or Metal Ions at pH 7.6 and 20 °C[a]

		Δ chem shift			
ATP	metal[b]	$-CH_2O-$	$-CH_2NH-$	$-CH_2NH-$	$-CH_2NH-$
+		−0.94	−0.09	−0.16	−0.94
–	Ca(II)	+0.02	0.00	−0.02	0.00
+	Ca(II)	−0.58	0.00	−0.01	−0.44
–	Mg(II)	−0.73	−0.08	+0.07	−0.56
+	Mg(II)	−0.44	−0.01	+0.20	−0.33
–	Zn(II)	+0.96	−0.11	−0.05	−1.04
+	Zn(II)	−0.72, −1.63	−0.37	−0.62	−0.90
–	Cd(II)[c]				
+	Cd(II)	−0.89	−0.04	−0.46	−0.07, −0.25, −0.86
–	La(III)	−0.21	−0.04	+0.05	−0.15
+	La(III)	−1.12	+0.09	−0.54	−0.99

[a] Chemical shifts are reported as Δ ppm compared to those for **1** (65.67, 46.40, 45.77, 44.46) at pH 7.6 and 20 °C. Two-dimensional ^{13}C–^1H spectral studies showed the $-CH_2O-$ (C2, 65.67 ppm) and $-CH_2NH-$ (C3, 46.40 ppm) and the other two $-CH_2NH-$ units (C5, 45.77 ppm; C6, 44.46 ppm) were short-range coupled and C3 and C5 were long-range coupled. [b] All metal ions were 0.015 M except lanthanum(III), which was 0.005 M. [c] The presence of a precipitate prevented the spectra from being obtained.

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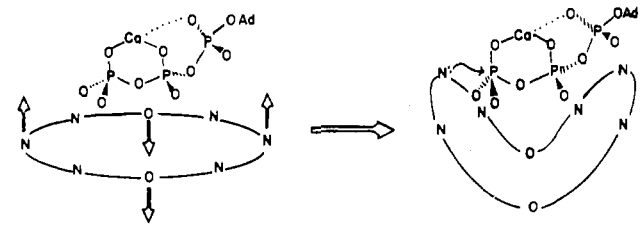
of both P_β (0.87 ppm) and P_γ (0.15 ppm) and no shift in P_α. This could indicate that the two terminal phosphates are the primary interactive sites of ATP with **1**. Care must be taken, however, in drawing definitive conclusions about binding from ^{31}P NMR data due to the sensitivity of the shifts to a variety of factors including pH and torsion angles. The intriguing aspect of the ^{31}P shifts for the ternary macrocycle–ATP–metal system is that for all three phosphorus atoms for calcium and magnesium, and for P_α and P_β for lanthanum, the chemical shifts are practically the same as in the presence of the binary ATP–metal system. Such a finding indicates that the metal potentially acts as proposed in the ATPase systems, i.e. by chelating to the ATP to bring it into a preferred conformation for interaction with the enzyme.[1,15] The anomalous P_γ shift of the lanthanide ion from the binary to ternary system may be a reflection of a different mode of binding of this metal with ATP in the presence and absence of macrocycle. Thus, the major "complexation" interaction of ATP is with the metal ion, and as a result, the chelated ATP is "wrapped" around the metal ion and is possibly more suitably shaped for insertion into the macrocyclic cavity compared to unbound ATP. That calcium(II) and lanthanum(III) have lessened affinity for nitrogen donor atoms is well established, and hence these metal ions can act to promote dephosphorylation without competitively binding to the macrocycle. This observation is further supported by the ^{13}C chemical shifts of **1**–metal mixtures, as discussed below. An additional factor in lanthanide ion assisted rate acceleration is its higher tripositive charge. On the other hand, the two metal ions, cadmium and zinc, that retard the dephosphorylation process elicit considerably different chemical shifts in the binary as opposed to the ternary ATP complexes. The rationale for these differences is explained on the basis of complexation interactions, which can be discerned from an examination of ^{13}C NMR data as discussed below.

An investigation of ^{13}C chemical shifts for the binary **1**–metal system (Table III) indicates that in the absence of ATP all of the metal ions, with the exception of calcium and to a lesser extent lanthanum, interact with the macrocycle. Only calcium ion does not affect the ^{13}C shifts of the macrocycle, while lanthanum promotes small shifts in the $-CH_2O-$ resonance and the high-field amine-associated resonance, assigned as the neutral amine. This nitrogen has been previously determined to be the central one in the triamine series.[7] The magnitude of the chemical shifts for the other metal ions indicates considerable interaction at two sites: the ether oxygen and the high-field neutral amine. The lack of dephosphorylation acceleration in the case of magnesium ion is thus understood in terms of the competitive binding of this metal to the macrocycle.

In the presence of the two metal ions that significantly inhibit the catalytic efficiency of **1**, zinc(II) and cadmium(II), complex ^{13}C NMR spectra are observed for the ternary associations. These include large additional shifts observed for the two low-field $-CH_2NH-$ resonances, assigned to the protonated ammonium nitrogens. In view of the large chemical shifts noted for **1** in the binary **1**–metal systems with zinc and cadmium, indicating strong interactions between the metals and macrocycle, the retardation in rates for these ternary macrocycle–ATP–metal systems is probably the result of competitive inhibition. These findings are further corroborated by the results of Martell, Lehn, and coworkers, who found that the tetraprotonated ligand forms complexes with cobalt(II), nickel(II), and zinc(II), resulting in displacement of protons from three of the protonated nitrogen atoms.[14] That large chemical shifts are still observed for ATP in the presence of metal–macrocycle complex, where dephosphorylation is minimal, can be explained if ion-pair formation is occurring between the positively charged metal–macrocycle complex and the negatively charged tripolyphosphate chain.

A clearer picture of the metal–ATP–macrocycle interactions can be obtained by examining the ^{13}C NMR data for binary macrocycle–ATP and ternary macrocycle–ATP–metal systems. In the absence of metal ion the interaction of ATP and **1** is evidenced by shifts of the ^{13}C resonances of almost 1 ppm for the $-CH_2O-$ resonance and the high-field $-CH_2NH-$ resonance. For

Scheme I



calcium, magnesium, and lanthanum ions with ATP in the presence of **1**, the major shifts are also the ether-associated carbons and the neutral amine carbons. At first glance the large shifts in the carbons associated with the neutral heteroatoms is puzzling, since the ATP–macrocycle interaction is anticipated to be largely through electrostatic and hydrogen-bonding interactions with the positively charged ammoniums. It is highly feasible, however, that the macrocyclic conformation does change on approach of the ATP molecule, from a relatively floppy circular form to a more structured receptacle, Scheme I. Repulsive interactions between the ether and incoming phosphate oxygens are anticipated, as well as nucleophilic attractions of the neutral amines for particularly the terminal phosphorus atom, resulting in the phosphorylated macrocycle **2**.

Phosphoramidate Formation. The first indication of the regulatory role of added metal ion is evidenced in the increased concentration of the phosphoramidate intermediate in the presence of magnesium(II), calcium(II), and lanthanum(III) in that order. For the pH range studied (3.6–9.0), the influence maximized at 7.6, but phosphoramidate is also observed when metal ion is present at pHs as low as 5.6. By comparison, the intermediate is not observed in the absence of metal ion in binary **1**–ATP systems much below a pH of 7. The maximum total percentage observed was 17% of the total phosphate species for **1** with ATP alone, 30%, 37%, and 47% for added magnesium, calcium, and lanthanum ions, respectively, for the concentrations as reported in Table I. The possibility of N- or O-coordinated or -associated phosphoramidate with the metal ion is appealing based on reports by Sargeson and co-workers.[23] Almost identical chemical shifts for the phosphoramidate phosphorus in the presence and absence of metal ion are observed, however, which indicates that interactions, if they occur, may be weak.

The nature of the metal ion influence on the intermediate phosphoramidate species was examined by observing the hydrolysis of the phosphorylated macrocycle **2** obtained from acetyl phosphate in the absence of nucleotide and other phosphate species. Rates in the absence and presence of calcium(II) were 0.097 and 0.054 min^{-1}, respectively, at pH 5.6 and 60 °C. Evidently at least one role of the metal ion, therefore, is in the stabilization of the intermediate phosphoramidate species **2**.

In the hydrolysis of phosphoramidates it has been established that $R_2NPO_3H^-$ is totally unreactive toward hydrolysis and that dephosphorylation apparently proceeds via metaphosphate elimination from $R_2NHPO_3^{2-}$.[24-27] Considering the Lewis acid nature of the metal ion and its propensity for complexing with the oxygens of the phosphoramide, the presence of a metal ion might be expected to increase the susceptibility of the phosphorus to nucleophilic attack, were an addition–elimination type mechanism in effect, and hence increase the hydrolytic rate. If the rate-determining step is elimination via a metaphosphate-type mechanism, however, the metal should compete with the protonated amine for the electron flow, slowing the hydrolysis rate, in agreement with the observed trends in this case. On the basis of the ^{13}P NMR data, which show virtually no shifts of the phosphoramidate resonance in the presence and absence of metal ion,

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however, an alternative explanation was sought. Metal ions can act to increase the acidity of the macrocyclic amines, as was noted for certain metal complexes in the presence of the tetraprotonated form of **1**, as cited earlier.[14] Given that pK_{a3} for **1** is between 7 and 8, and that the presence of the electron-withdrawing $-PO_3^{2-}$ portion of the phosphoramidate would certainly make the amidate proton more acidic than the other protonated nitrogens in the macrocycle, it is reasonable to suggest that, at a pH of 5.6, a greater percentage of the phorphoramidate is actually in the deprotonated and unreactive form. This hypothesis is supported by the observed correlation of increased phosphoramidate formation (or stabilization) with increasing effective nuclear charge (or Lewis acidity) of the metal ion Mg(II) < Ca(II) < La(III).

Pyrophosphate Formation. In the presence of metal ions, the formation of pyrophosphate was observed, the net result of the metal ion "regulation". When the pH of a solution of **1**–ATP–Ca(II) was adjusted to 4 to solubilize precipitated calcium salts, pyrophosphate was observed in less than 24 h at room temperature. Without metal ion, pyrophosphate was not observed under similar conditions. The formation of pyrophosphate appears to be contingent upon reaction of the phosphoramide with inorganic phosphate present in solution.[7,8] Thus the increased concentration of the intermediate provided by magnesium(II), calcium(II), and lanthanum(III) promotes the phosphorylation reaction, provided the phosphoramidate is accessible to incoming phosphate. Indeed, the ditopic nature of **1** is such that an additional binding site is available for inorganic phosphate present in solution. The imposed proximity of the two reacting species can be considered as activation toward the transition state. A similar mechanism has been proposed by Hosseini and Lehn in the observation of pyrophosphate formation in the catalysis of acetyl phosphate dephosphorylation by **1**.[7] In that study pyrophosphate formation was inhibited by the addition of anionic species including nucleotides. Thus, removal of the nucleotides competing with incoming phosphate for the macrocyclic cavity by complexation with the metal ion may well be a factor in enhancing the rate of pyrophosphate formation.

Conclusions. In progressing from binary ATP–**1** to a ternary association by the addition of metal ions, a more complex and challenging mechanistic problem is obtained. The broad range of dephosphorylation rates includes both retardation and acceleration depending on the metal ion, as well as stabilization of the phosphoramidate intermediate and pyrophosphate synthesis for magnesium(II), calcium(II), and lanthanum(III). Increases in rate are attributed to the role of the metal ion in "structuring" the ATP molecule for a better fit of the two terminal phosphates in the macrocyclic cavity. Spectroscopic evidence indicates that the macrocycle undergoes considerable conformation changes upon reaction with ATP, which are proposed to result from repulsive interactions between the phosphate and ether oxygen atoms and attraction of the nucleophilic neutral amines by P_γ. The rate retardations observed for zinc(II) and cadmium(II) are primarily attributed to their propensity for complexation with **1**, providing competitive inhibition of the catalytic process. The stabilization of the phosphoramidate intermediate by the magnesium, calcium, and lanthanum ions is probably best explained by deprotonation of the phosphoramidate nitrogen in the presence of the metal ion. The results of complexation studies between the binary macrocycle–polyphosphate and ternary macrocycle–polyphosphate–metal ion systems will be reported in a subsequent paper.

Acknowledgment. The authors thank Professor Jean-Marie Lehn and Dr. Mir Wais Hosseini for their helpful discussions and Eduardo Veliz for his technical assistance in obtaining the 2-D NMR. This work was supported by a grant from the Institute of General Medical Sciences (No. GM 33922) of the National Institutes of Health.

Registry No. **1**, 43090-52-4; PP, 14000-31-8; PPP, 14127-68-5; ATP, 56-65-5; ADP, 58-64-0; ATPase, 9000-83-3; Mg, 7439-95-4; Ca, 7440-70-2; La, 7439-91-0; Zn, 7440-66-6; Cd, 7440-43-9.

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Binuclear Copper Complexes of Ligands Providing Three Donors to Each Metal Ion

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Received October 16, 1986

The synthesis of 2,6-bis{[2-(1-pyrazolyl)ethyl]methylamino}-*p*-cresol and its 3-*tert*-butylpyrazole derivative are described. These new chelating agents are able to provide two nitrogen donors to each metal ion in addition to a phenolate group, which bridges the metal atoms. The syntheses of the binuclear Cu(II)–azide and Cu(I)–carbonyl complexes are presented. The copper(II) dimer is diamagnetic at room temperature, and the singlet–triplet transition energy is greater than 2000 cm^{-1}. The Cu(I) dimer reacts with dioxygen irreversibly even with the steric hindrance provided by the *tert*-butyl group.

The synthesis of ligands capable of binding two metal ions in a proximal relationship continues to attract the attention of both organic and inorganic chemists.[2-12] Our interest in this area results from the need for chelates able to bind two copper ions in a manner that mimics the active site in the type III copper proteins, most notably hemocyanin and tyrosinase.[13] While many such ligands exist, very few form stable binuclear copper(I) adducts, a prerequisite for probing reversible binding of dioxygen. We report here the synthesis of two ligands, Hpeac (**1a**) and H(*t*-Bu)peac (**1b**), and their binuclear Cu(II) (**8**) and Cu(I) (**9**) derivatives.

Experimental Section

All reagents and solvents were purchased from commercial sources and used as received. 2,6-Diacetamido-*p*-cresol (**2**) was prepared by the literature method.[2] Melting points were obtained with use of a Fisher–

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